J|A|C|S
ARTICLES
Published on Web 02/02/2006

Preparation and Evaluation of Unimolecular Pentavalent and Hexavalent Antigenic Constructs Targeting Prostate and Breast Cancer: A Synthetic Route to Anticancer Vaccine Candidates

Govind Ragupathi,† Fusataka Koide,† Philip O. Livingston,† Young Shin Cho,‡ Atsushi Endo,‡ Qian Wan,‡ Maria K. Spassova,§ Stacy J. Keding,‡ Jennifer Allen,‡ Ouathek Ouerfelli,§ Rebecca M. Wilson,‡ and Samuel J. Danishefsky*,‡,⊥

Contribution from the Laboratory of Tumor Vaccinology, Clinical Immunology Service, Department of Medicine, the Laboratory for Bioorganic Chemistry, and the Organic Synthesis Core Laboratory, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10021, and Department of Chemistry, Columbia University, Havemeyer Hall, 3000 Broadway, New York, New York 10027

Received October 24, 2005; E-mail: s-danishefsky@ski.mskcc.org

Abstract: Several novel, fully synthetic, carbohydrate-based antitumor vaccines have been assembled. Each construct consists of multiple cancer-related antigens displayed on a single polypeptide backbone. Recent advances in synthetic methodology have allowed for the incorporation of a complex oligosaccharide terminating in a sialic acid residue (i.e., GM2) as one of the carbohydrate antigens. Details of the vaccine synthesis as well as the results of preliminary immunological investigations are described herein.

Introduction

A long-standing ambition of cancer therapeutics has been the development of a means to exploit the considerable resources of the immune system for the eradication of micrometastatic cancer. Appealing as this strategy may appear, the realization of an effective antitumor vaccine is fraught with challenges. Antibody induction in this context is difficult because most tumor antigens are self-antigens or slightly modified versions. In addition, tumor cells have been shown to set into motion a number of mechanisms that serve to depress the host immune system. Despite these and other challenges, preclinical and clinical studies demonstrating that naturally acquired, actively induced, or passively administered antibodies can eliminate circulating tumor cells and micrometastatic disease have established the antibody approach to cancer therapy to be worthy of serious pursuit.[1]

Because transformed cancer cells exhibit abnormal cell surface glycosylation patterns, carbohydrates represent a potentially useful class of antigens. Indeed, patients with natural or vaccine-induced antibodies against GM2, STn, and a number of other cell surface carbohydrate-based antigens have been reported to survive longer than patients lacking these antibodies.[2]

In this context, our laboratories have a longstanding program directed toward the preparation and biological evaluation of synthetic carbohydrate-based antitumor vaccines. Experience has taught us that the optimal approach to antibody induction is through covalent attachment of a glycopeptide construct to an immunogenic carrier molecule, such as keyhole limpet hemocyanin (KLH),[3] and the use of a potent saponin immunological adjuvant, such as QS-21.[4] To date, a number of our fully synthetic carbohydrate constructs have been shown to induce both IgG and IgM responses which react with tumor cells expressing the corresponding antigens. *In this paper, we describe studies which started with questions of synthetic organic chemistry and are now at the stage of evaluation in clinical settings.*

Our initial investigations focused on synthetic monovalent glycopeptide constructs, in which a single carbohydrate antigen is attached to an immunogenic carrier molecule.[5] While monovalent vaccines have shown promise in early clinical

† Laboratory of Tumor Vaccinology, Clinical Immunology Service, Department of Medicine, Sloan-Kettering Institute for Cancer Research.
‡ Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research.
§ Organic Synthesis Core Laboratory, Sloan-Kettering Institute for Cancer Research.
⊥ Columbia University.

(1) La Poole, I. C.; Gerberi, M. A. T.; Kast. W. M. *Curr. Opin. Oncol.* 2002, *14*, 641–648.
(2) (a) Livingston, P. O.; Wong, G. Y. C.; Adluri, S.; Tao, Y.; Padavan, M.; Parente, R.; Hanlon, C.; Calves, M. J.; Helling, F.; Ritter, G.; Oettgen, H. F.; Lloyd, K. O. *J. Clin. Oncol.* 1994, *12*, 1036–1044. (b) MacLean, G. D.; Reddish, M. A.; Koganty, R. R.; Longenecker, B. M. *J. Immunotherapy* 1996, *19*, 59–68.
(3) (a) Helling, F.; Shang, A.; Calves, M.; Zhang, S. L.; Ren, S. L.; Yu, R. K.; Oettgen, H. F.; Livingston, P. O. *Cancer Res.* 1994, *54*, 197–203. (b) Helling, F.; Zhang, S.; Shang, A.; Adluri, S.; Calves, M.; Koganty, R.; Longenecker, B. M.; Yao. T. J.; Oettgen, H. F.; Livingstoo, P. O. *Cancer Res.* 1995, *55*, 2783–2788.
(4) (a) Kensil, C. R.; Patel, U.; Lennick, M.; Marciani, D. *J. Immunol.* 1991, *146*, 431–437. (b) Marciani, D. J.; Press, J. B.; Reynolds, R. C.; Pathak, A. K.; Pathak, V.; Gundy, L. E.; Farmer, J. T.; Koratich, M. S.; May, R. D. *Vaccine* 2000, *18*, 3141–3151. (c) Kim, S.-K.; Ragupathi, G.; Musselli, C.; Choi, S. J.; Park, Y. S.; Livingston, P. O. *Vaccine* 1999, *18*, 597–603. (d) Kim, S.-K.; Ragupathi, G.; Cappello, S.; Kagan, E.; Livingston, P. O. *Vaccine* 2000, *19*, 530–537.

10.1021/ja057244+ CCC: $33.50 © 2006 American Chemical Society

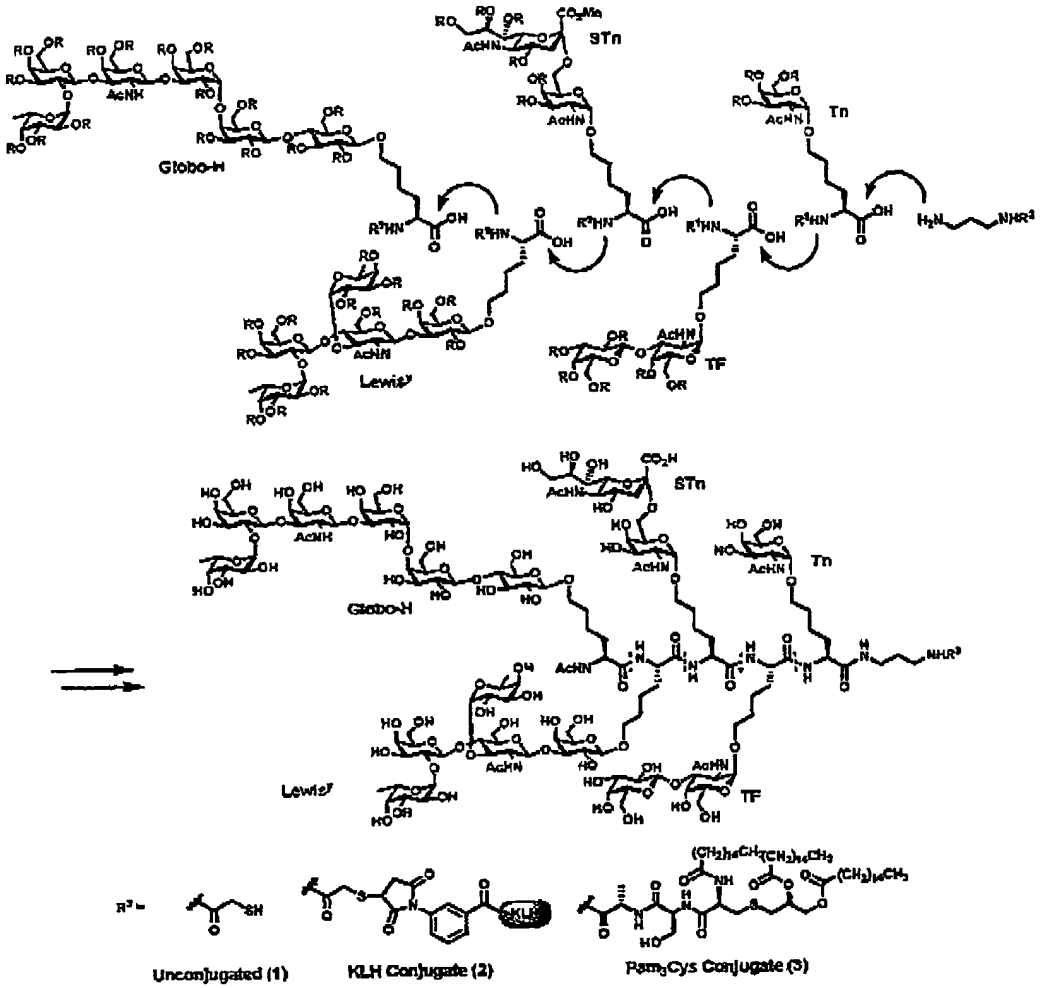


Figure 1. Structure of unimolecular pentavalent vaccine.

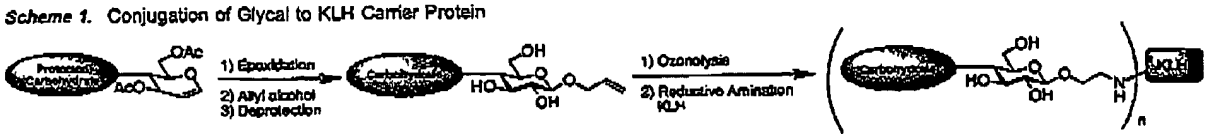
settings, this approach fails to take account of the actual degree of heterogeneity of carbohydrate epitopes expressed on a transformed cell surface.[6] We postulated that by combining several carbohydrate antigens closely associated with a particular cancer type, we could induce a more robust immune response, decreasing the percentage of tumor cells that can evade an immunological response. One can envision two implementations of the polyvalent antigen strategy. In the polyvalent monomeric approach, mixtures of different monovalent-KLH constructs would be injected simultaneously in the hopes of evoking an antibody response to each carbohydrate antigen. Indeed, we have demonstrated that mixtures of monovalent-KLH conjugates injected as tetravalent or heptavalent vaccines lead to induction of antibody titers against each individual antigen at levels comparable to those achieved with the individual monovalent vaccines.[7] This approach, however, suffers from several serious limitations. First, the polyvalent monomeric strategy requires the use of increased levels of carrier protein. In addition, regulatory requirements would necessitate the validation of each individual component of the vaccine mixture. Finally, the synthesis of each monovalent-KLH construct involves a low-yielding final conjugation step.

(5) (a) Ragupathi, G.; Park, T. K.; Zhang, S. L.; Kim, I. J.; Graber, L.; Adluri, S.; Lloyd, K. O.; Danishefsky, S. J.; Livingston, P. O. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 125–128. (b) Slovin, S. F.; Ragupathi, G.; Adluri, S.; Ungers, G.; Terry, K.; Kim, S.; Spassova, M.; Bornmann, W. G.; Fazzari, M.; Dantis, L.; Olkiewicz, K.; Lloyd, K. O.; Livingston, P. O.; Danishefsky, S. J.; Scher, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5710–5715. (c) Gilewski, T.; Ragupathi, G.; Bhuta, S.; Williams, L. J.; Musselli, C.; Zhang, X.-F.; Bencsath, K. P.; Panageas, K. S.; Chin, J.; Hudis, C. A.; Norton, L.; Houghton, A. N.; Livingston, P. O.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3270–3275. (d) Krug, L. M.; Ragupathi, G.; Hood, C.; Kris, M. G.; Miller, V. A.; Allen, J. R.; Keding, S. J.; Danishefsky, S. J.; Gomez, J.; Tyson, L.; Pizzo, B.; Baez, V.; Livingston, P. O. *Clin. Cancer Res.* **2004**, *10*, 6094–6100. (e) Sabbatini, P. J.; Kudryashov, V.; Ragupathi, G.; Danishefsky, S. J.; Livingston, P. O.; Bornmann, W.; Spassova, M.; Zatorski, A.; Spriggs, D.; Aghajanian, C.; Soignet, S.; Peyton, M.; O'Flaherty, C.; Curtin, J.; Lloyd, K. O. *Int. J. Cancer.* **2000**, *87*, 79–85.

(6) (a) Zhang, S. L.; Cordon-Cardo, C.; Zhang, H. S.; Reuter, V. E.; Adluri, S.; Hamilton, W. B.; Lloyd, K. O.; Livingston, P. O. *Int. J. Cancer* **1997**, *73*, 42–49. (b) Zhang, S. L.; Zhang, H. S.; Cordon-Cardo, C.; Reuter, V. E.; Singhal, A. K.; Lloyd, K. O.; Livingston, P. O. *Int. J. Cancer* **1997**, *73*, 50–56.

(7) (a) Ragupathi, G.; Cappello, S.; Yi, S. S.; Canter, D.; Spassova, M.; Bornmann, W. G.; Danishefsky, S. J.; Livingston, P. O. *Vaccine* **2002**, *20*, 1030–1038. (b) Ragupathi, G.; Koide, F.; Sathyan, N.; Kagan, E.; Spassova, M.; Bornmann, W.; Gregor, P.; Reis, C. A.; Clausen, H.; Danishefsky, S. J.; Livingston, P. O. *Cancer Immunol. Immunother.* **2003**, *52*, 608–616.

Scheme 1. Conjugation of Glycal to KLH Carrier Protein



For these and other reasons, we prefer the unimolecular multivalent scenario, wherein several different carbohydrate antigens are displayed on a single polypeptide backbone, which would require only one conjugation step. In this context, we previously disclosed the synthesis of a unimolecular trivalent vaccine construct, which induced antibodies against each of the component antigens.[8] More recently, we disclosed the syntheses of unconjugated (1), KLH-conjugated (2), and PamCys-conjugated (3) unimolecular pentavalent constructs displaying the Globo-H, Le^y, STn, TF, and Tn antigens, each of which is known to be overexpressed on prostate and breast cancer cell surfaces.[9]

As shown in Figure 1, our global strategy for the preparation of the multivalent construct required us to assemble a pool of appropriately protected glycosylamino acids, which would then be iteratively coupled to form the fully glycosylated polypeptide backbone. The design and implementation of our synthetic route would necessitate careful consideration of issues of polypeptide stereochemical homogeneity and protecting group logic.

Our protecting group strategy required the carbohydrate sectors of the pre-assembled glycosylamino acid cassettes to be completely protected (cf. R) throughout the coupling sequence. We elected to block the N-termini (cf. R^1) of the cassettes with the fluorenylmethyl carbonate (Fmoc) protecting group so that the coupling sequence would consist of iterative peptide couplings followed by Fmoc deprotections of the N-termini. The amino acid presenting the Tn antigen would ultimately be linked to the carrier molecule and must possess a differentially protected partial linker. We chose to append a Boc-protected (cf. R^2) diaminopropyl unit to the C-terminus of the Tn cassette, which would be further elaborated following assembly of the pentapeptide backbone.

We now turn briefly to the methods employed in preparing the individual glycosylamino acid building blocks. Our carbohydrate assembly method provides a terminal glycal unit as a functional handle, which serves as the site of appendage of the carbohydrate to the appropriate amino acid residue or carrier protein. The first-generation monovalent vaccine constructs prepared in our laboratory were composed of carbohydrate epitopes conjugated directly to the KLH carrier molecule.[10] Conjugation was typically achieved by functionalization of an allyl glycoside, itself obtained through glycal epoxidation followed by treatment with allyl alcohol (Scheme 1). Thus, ozonolysis of the terminal olefin of the allyl glycoside provides the aldehyde, which is then appended to the lysine residues of the KLH carrier protein through reductive amination. Despite its general utility, this method of conjugation suffers several drawbacks. Most importantly, functionalization of the glycal to the allyl ether must be accomplished at the fairly late peracetate stage of the carbohydrate synthesis, due to the instability of the allyl glycoside to the dissolving metal conditions required for global deprotection. The glycal epoxidation step typically proceeds with only moderate levels of stereoselectivity, resulting in a loss of material at a very late stage in the carbohydrate synthesis.

Despite its imperfections, this original protocol has served as the basis of the three general methods that we currently rely on for the preparation of our glycosylamino acid cassettes (Figure 2). As will be seen, each of these protocols possesses its unique strengths and shortcomings. The first method (*a*), which relies heavily upon the precedent of the allyl glycoside ozonolysis protocol, commences with the more stable pentenyl glycoside.[8a] Ozonolysis of the terminal olefin is followed by Horner–Emmons reaction to yield the dehydroamino acid, as shown. Enantioselective reduction of the olefin, followed by standard functional group manipulations, affords the requisite glycosylamino acid cassette. The stability of the pentenyl glycoside allows for functionalization of the glycal at a much earlier stage of the synthesis in comparison with the allyl ether protocol described above. Thus, the overall impact of the chemical loss accompanying the glycal epoxidation step is significantly less pronounced. A liability of this ozonolysis method lies in the fact that the amino acid stereocenter must be installed through an enantioselective reduction following coupling to the carbohydrate fragment.

The second commonly employed method (*b*)[11] commences with the appropriately protected allyl glycoside. This intermediate is subjected to olefin cross-metathesis with protected allyl glycine in the presence of ruthenium catalyst. Reduction of the resultant olefin with concurrent benzyl ester cleavage provides the glycosylamino acid cassette. Because the allyl glycine is commercially available in enantiomerically pure form, this method does not require the installation of a stereocenter. However, this protocol does suffer from the requirement that the allyl ether be installed from the glycal at the relatively late peracetate stage of the carbohydrate synthesis.

Finally, we have developed a protocol (*c*)[12] that allows for the introduction of the amino acid functionality directly from glycal epoxide or trichloroacetimidate donors through coupling with hydroxynorleucine in the presence of Lewis acid. This direct method allows for appendage of the amino acid residue containing the requisite stereocenter in one coordinated step; however, the efficiency of the protocol is still limited by the moderate stereoselectivity of the glycal epoxidation and by the need to synthesize hydroxynorleucine. It will be noted that, although we have yet to develop the ideal protocol for glycosylamino acid assembly, each of these three methods ultimately allows for the introduction of the amino acid moiety

(8) (a) Allen, J. R.; Harris, C. R.; Danishefsky, S. *J. J. Am. Chem. Soc.* **2001**, *123*, 1890–1897. (b) Ragupathi, G.; Coltart, D. M.; Williams, L. J.; Koide, F.; Kagan, E.; Allen, J.; Harris, C.; Glunz, P. W.; Livingston, P. O.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13699–13704.
(9) Keding, S. J.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11937–11942.
(10) Park, T. K.; Kim, I. J.; Hu, S. H.; Bilodeau, M. T.; Randolph, J. T.; Kwon, O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1996**, *118*, 11488–11500.
(11) Biswas, K.; Coltart, D. M.; Danishefsky, S. J. *Tetrahedron Lett.* **2002**, *43*, 6107–6110.
(12) (a) Keding, S. J.; Endo, A.; Biswas, K.; Zatorski, A.; Coltart, D. M.; Danishefsky, S. J. *Tetrahedron Lett.* **2003**, *44*, 3413–3416. (b) Keding, S. J.; Endo, A.; Danishefsky, S. J. *Tetrahedron* **2003**, *59*, 7023–7031.

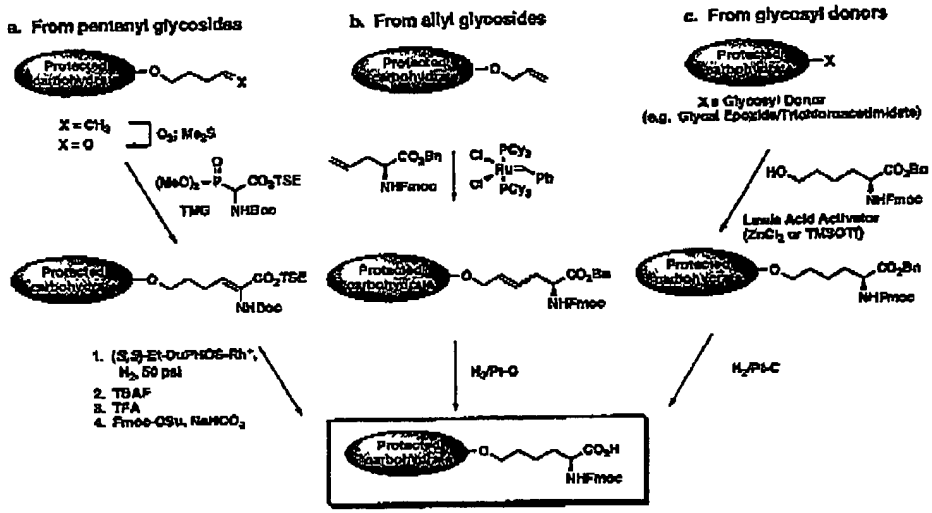


Figure 2. Methods used for the preparation of the glycosylamino acids.

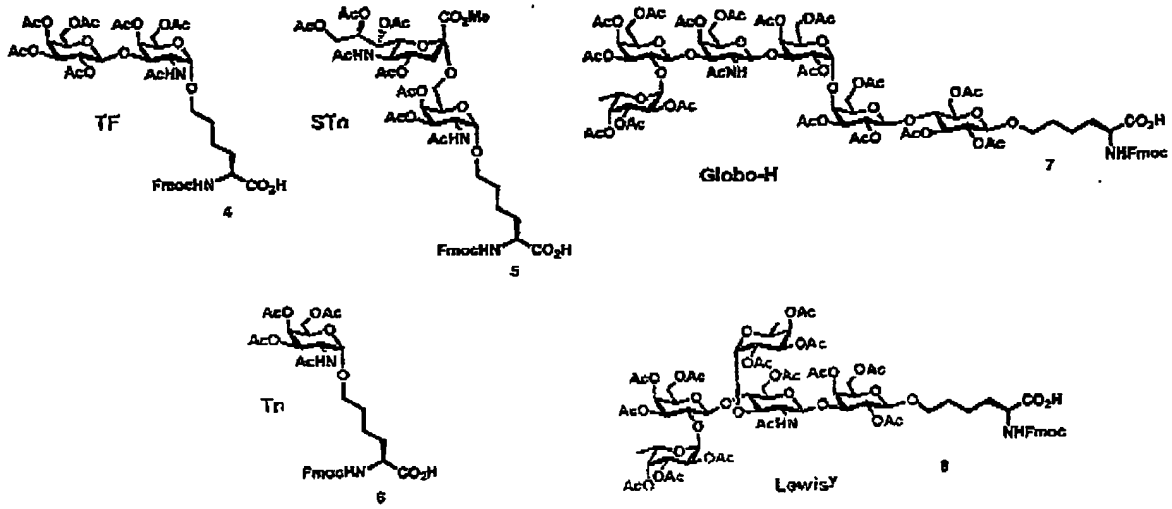


Figure 3. Fully protected glycosylamino acids.

in a stereochemically defined manner to provide a glycosylamino acid in which the carbohydrate is separated from the peptide backbone by a four-carbon linker.

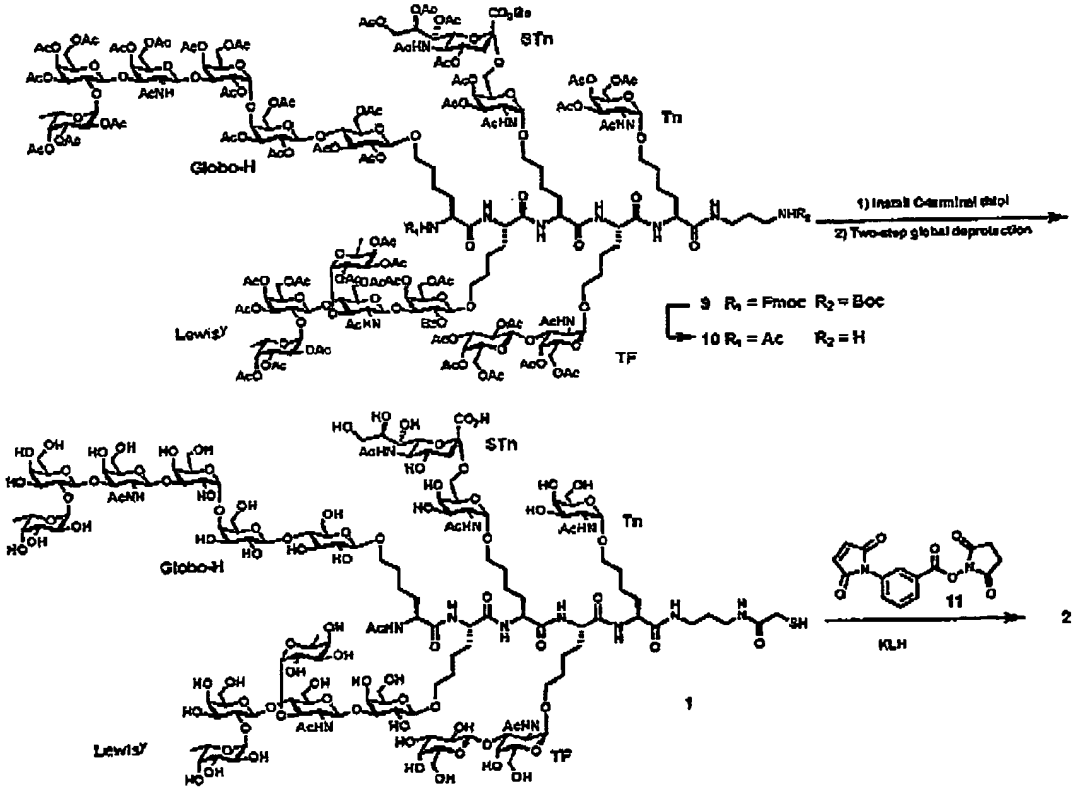
Results and Discussion

In fashioning our pool of glycosylamino acids for the pentavalent vaccine, we employed two of the three methods described above. Thus, the α-linked glycosylamino acids—TF (4), STn (5), and Tn (6)—and the Le^y cassette (8) were prepared according to the hydroxynorleucine approach (*c*), and the Globo-H cassette (7) was obtained through the cross-metathesis protocol (*b*) (Figure 3).

With the fully protected glycosylamino acids in hand, we were prepared to construct the pentapeptide backbone. Thus, through iterative deprotection and coupling reactions, glycopeptide 9 was assembled in 28% overall yield for nine transformations (Scheme 2). It will be noted that, at this point, the N-terminus, the C-terminal partial linker, and the carbohydrate domain of glycopeptide 9 were each equipped with orthogonally removable protecting groups. We next selectively removed the N-terminal Fmoc blocking group and replaced it with an acetyl cap. Subsequent cleavage of the C-terminal Boc protecting group gave rise to intermediate **10**. At this point, the routes to the KLH conjugate (**2**) and the PamCys conjugate (**3**) diverged. The preparation of the former required appendage of a thiol functionality to the partial linker. A two-step global deprotection of the carbohydrate domain then gave rise to the unconjugated glycopeptide **1**. Conjugation to the carrier protein was achieved through derivatization of KLH with maleimide **11**, followed by Michael addition of the thiol to the maleimide handle, affording construct **2**, with a glycopeptide-to-protein ratio of 228:1.

The formation of the PamCys conjugate from intermediate **10** commenced with a series of peptide coupling and deprotection reactions to install alanine and serine amino acids. A global deprotection sequence was followed by treatment with the Pam_3Cys pentafluorophenyl ester to afford the PamCys conjugate **3**.

Scheme 2



For reasons that will soon be made clear, the results of biological studies with the pentavalent vaccines **1**, **2**, and **3** led us to pursue the syntheses of two additional multivalent constructs: a modified unimolecular pentavalent glycopeptide, **12**, and the first hexavalent glycopeptide, **13** (Figure 4). In the pentavalent construct (**12**), the Le^y antigen is replaced with the GM2 antigen, while the hexavalent vaccine (**13**) displays both the GM2 and Le^y antigens. Several factors influenced our decision to incorporate the GM2 antigen into our unimolecular construct. For one, this antigen is overexpressed on the cell surfaces of a number of human cancers, including breast cancer and prostate cancer.[2a,13] In addition, GM2-induced antibodies have been shown to be active against human GM2-positive cells, and human clinical trials conducted with GM2 alone have demonstrated a correlation between enhanced GM2 antibody levels and survival.

In designing our modified constructs, we decided to position GM2, a terrasaccharide antigen, next to STn. In doing so, we would allow for the sequential assembly of the glycopeptide in order of carbohydrate size, from the smallest (Tn) to the largest and most complex (Globo-H), thus minimizing loss of the more difficultly prepared carbohydrates. Furthermore, for synthetic convenience, we elected to extend the distance between the Globo-H and GM2 residues and the peptide backbone through the use of a six-carbon linker (Figure 5).

The global strategy for the preparation of **12** and **13** is analogous to that applied in the preparation of the first pentavalent vaccine construct. Thus, the STn (**5**), Tn (**6**), TF (**4**), and Le^y (**8**) glycosylamino acids were prepared according to the methods described above. The chain-extended GM2 glycosylamino acid (**14**) and Globo-H glycosylamino acid (**15**) were prepared according to a modified cross-metathesis protocol, in which the pentenyl glycoside, rather than the allyl glycoside, undergoes a cross-metathesis coupling, followed by olefin reduction.[14]

With the requisite glycosylamino acids in hand, we were prepared to assemble the modified pentavalent glycopeptide construct (**12**). The Tn glycosylamino acid (**6**) was coupled with *tert*-butyl *N*-(3-aminopropyl)carbamate, which serves as a partial linker (Scheme 3). This unit (**16**) was then elongated to the pentapeptide **20** via iterative Fmoc deprotection and coupling reactions. Our slightly modified coupling conditions allowed us to access **20** in 57% overall yield for nine transformations. The five-step sequence from intermediate **20** to the conjugation precursor **12** proceeded in 68% overall yield.

The preparation of the hexavalent glycopeptide commenced with tetravalent intermediate **19**, which was prepared in significant quantities (up to 0.12 g) en route to the pentavalent construct. Iterative coupling of the Le^y and Globo-H glycosylamino acids, followed by the five-step preconjugation sequence, afforded the hexavalent glycopeptide **13** (Scheme 4). We note that the syntheses of these complex constructs are quite

(13) (a) Livingston, P. O.; Natoli, E. J.; Calves, M. J.; Stockert, E.; Oettgen, H. F.; Old, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2911–2915.

(14) (a) Wan, Q.; Cho, Y. S.; Lambert, T. H.; Danishefsky, S. J. *J. Carbohydr. Chem.* **2005**, *24*, 425–440. (b) Cho, Y. S.; Wan, Q.; Danishefsky, S. J. *Bioorg. Med. Chem.* **2005**, *13*, 5259–5266.

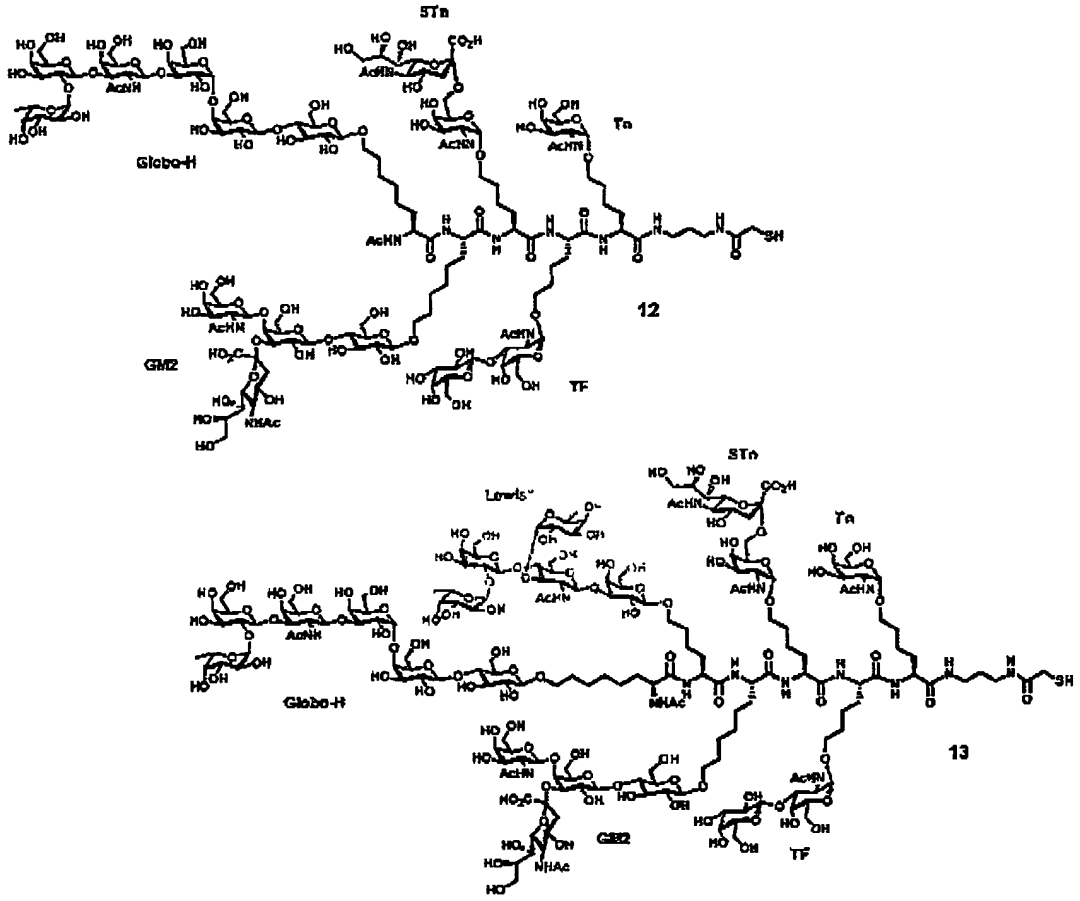


Figure 4. Modified pentavalent and hexavalent vaccine targets.

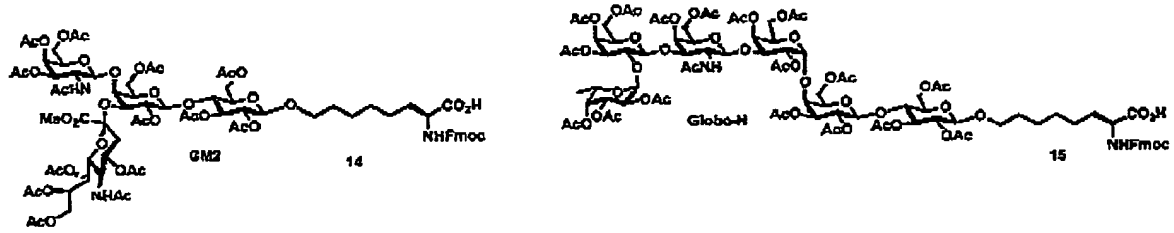


Figure 5. Chain-extended glycosylamino acids.

scalable, allowing access to up to 80 mg of 12 and up to 12 mg of 13.[15]

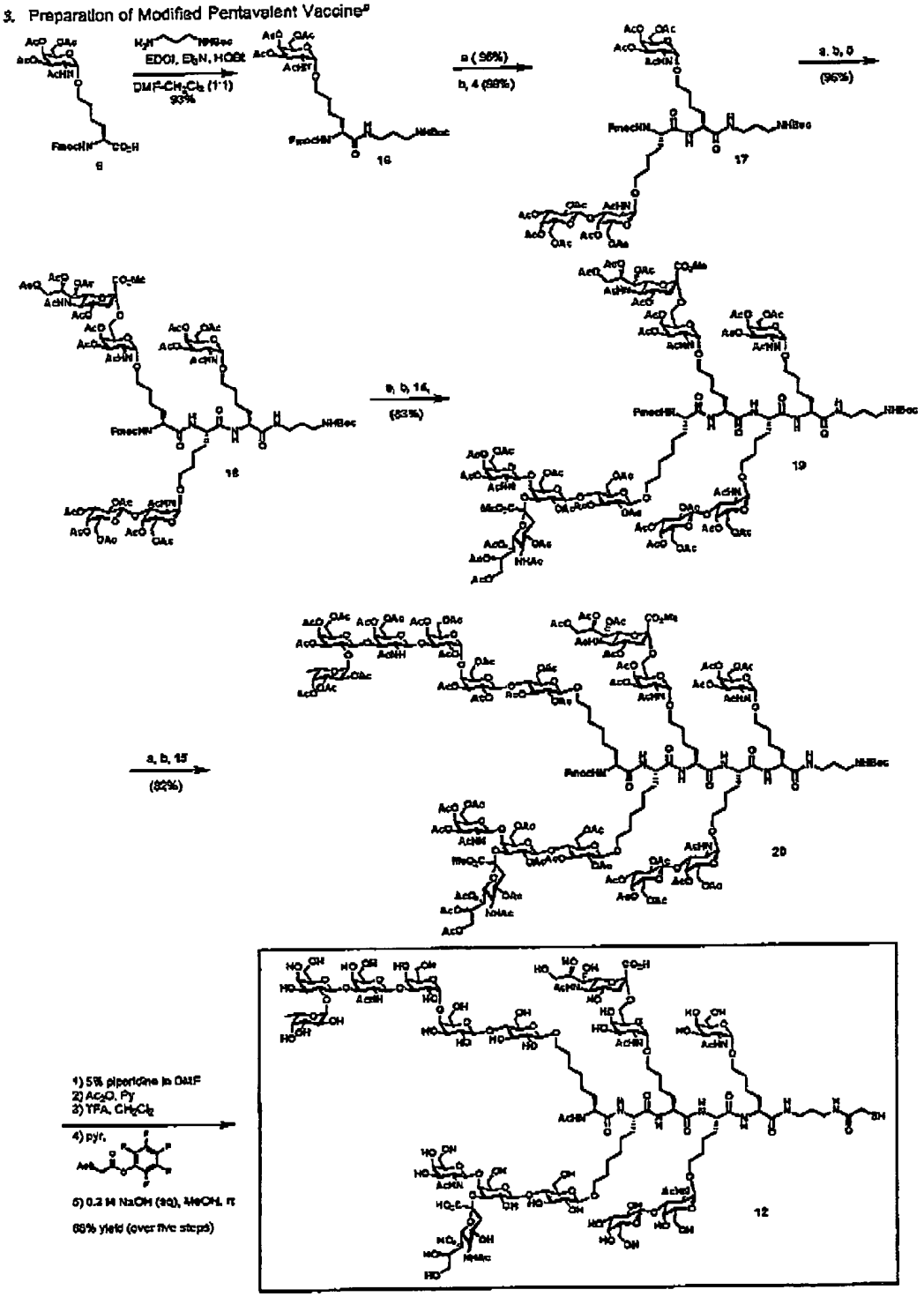
Biological Evaluations

The immunogenicity of the first-generation unimolecular pentavalent vaccine (1−3) was evaluated in comparison with that of the corresponding pooled monovalent vaccines. Groups of five mice were immunized three times at one-week intervals with (a) KLH-conjugated pentavalent vaccine (2) (10 μg of pentavalent construct conjugated to KLH), (b) PamCys-conjugated pentavalent vaccine (3) (30 μg of pentavalent construct conjugated to PamCys), (c) unconjugated pentavalent vaccine (1) (10 μg), (d) unconjugated pentavalent vaccine (1) (10 μg) + KLH, or (e) the pooled monovalent vaccines corresponding to each of the antigens displayed on the unimolecular construct, each conjugated to KLH (3 μg of each construct conjugated to KLH). All vaccines included 10 μg of QS-21 adjuvant and were administered subcutaneously over the lower abdomen.

Following completion of the immunization regimen, enzyme-linked immunosorbent assays (ELISA) were performed to determine the IgM and IgG serum antibody titers achieved for each group of mice (Table 1 and Figure 6). The unconjugated

(15) We note that, in each synthesis, the Globo-H glycosylamino acid was the limiting reagent.

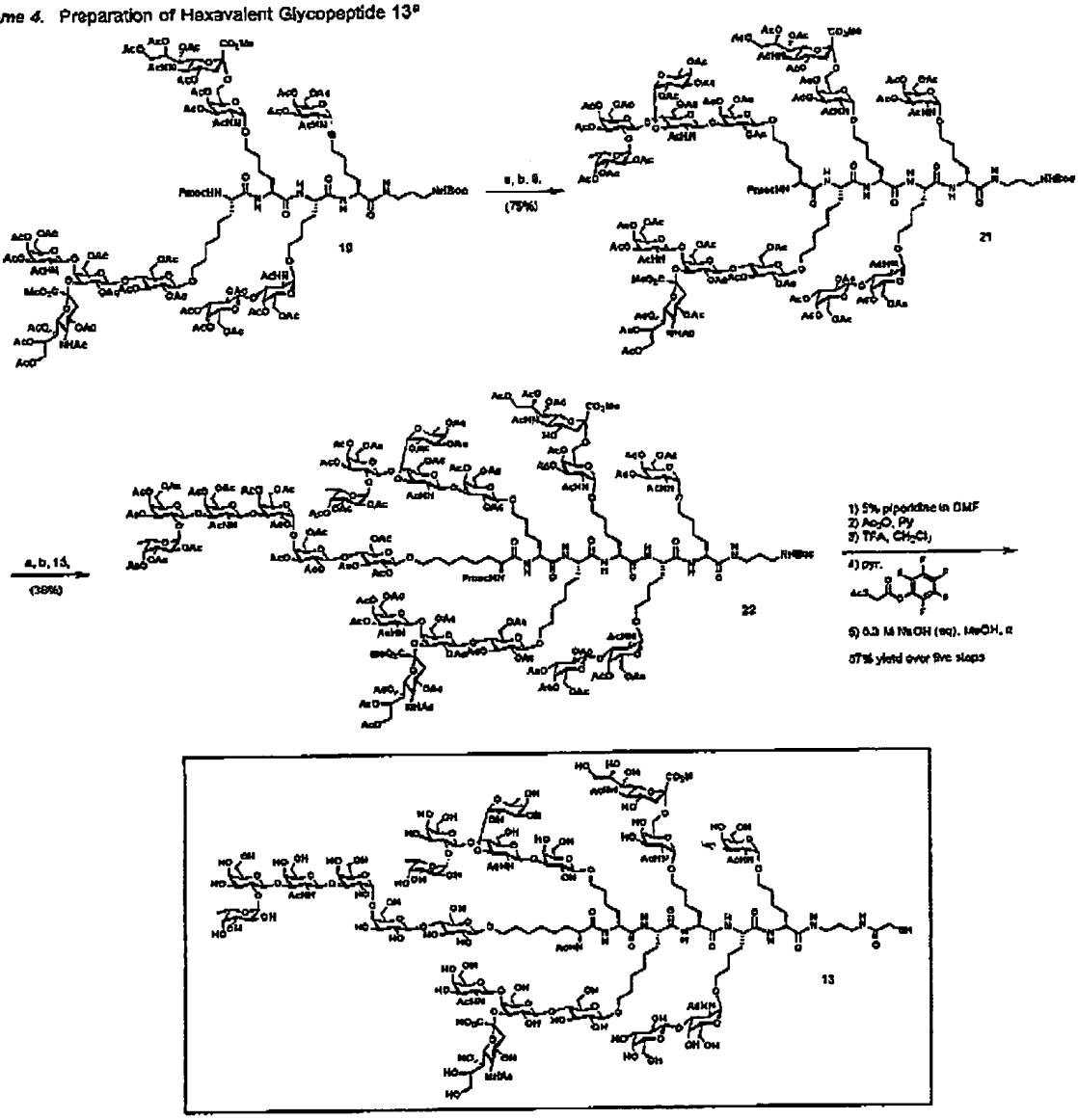
Scheme 3. Preparation of Modified Pentavalent Vaccine[a]



[a] Key: (a) 5% piperidine in DMF; (b) EDCI, HOBt, DMF–CH_2Cl_2 (1:1).

pentavalent vaccine (**1**), either alone or mixed with KLH, generally induced lower titers than did the KLH-conjugated vaccine. While the PamCys conjugate (**3**) was quite immunogenic, the titers were still somewhat lower than those observed for the KLH conjugate (**2**). Evaluation of the pooled monovalent vaccines reveals a notably superior IgM response

Scheme 4. Preparation of Hexavalent Glycopeptide 13[a]



[a] Key: (a) 5% piperidine in DMF; (b) EDCI, HOBt, DMF–CH_2Cl_2 (1:1).

against Tn, but a much lower response against the Globo-H ceramide and other antigens, in comparison with the response obtained with pentavalent-KLH conjugate (2). These results suggest that the positioning of the antigen on the multivalent construct may play a role in determining its immunogenicity. It is of note that only a minimal antibody response was observed against the Le^y ceramide. The low response against Le^y is not entirely unexpected, since compounds that are endogenously expressed at high levels, such as Le^y, are typically less effective as antigens than are those which are naturally present only in low levels.

The cell surface reactivity of the vaccine-induced antibodies was determined by Fluorescent Activated Cell Sorter (FACS) assay analysis with three different cell lines, each expressing high levels of two or more of the antigens of interest (Table 2). Each of the vaccines that induced antibodies against individual antigens also induced antibodies reactive with one or more cell lines expressing these antigens. The flow cytometry results for the five mice receiving the unimolecular pentavalent-KLH vaccine (2) are shown in Figure 7. Both the pool of monovalent vaccines and the unimolecular pentavalent vaccine induced antibodies that reacted significantly with all three cell lines.

Conclusion

In summary, we have described herein the most complex totally synthetic vaccines ever readied for human clinical evaluations. The individual complex carbohydrate-based anti-

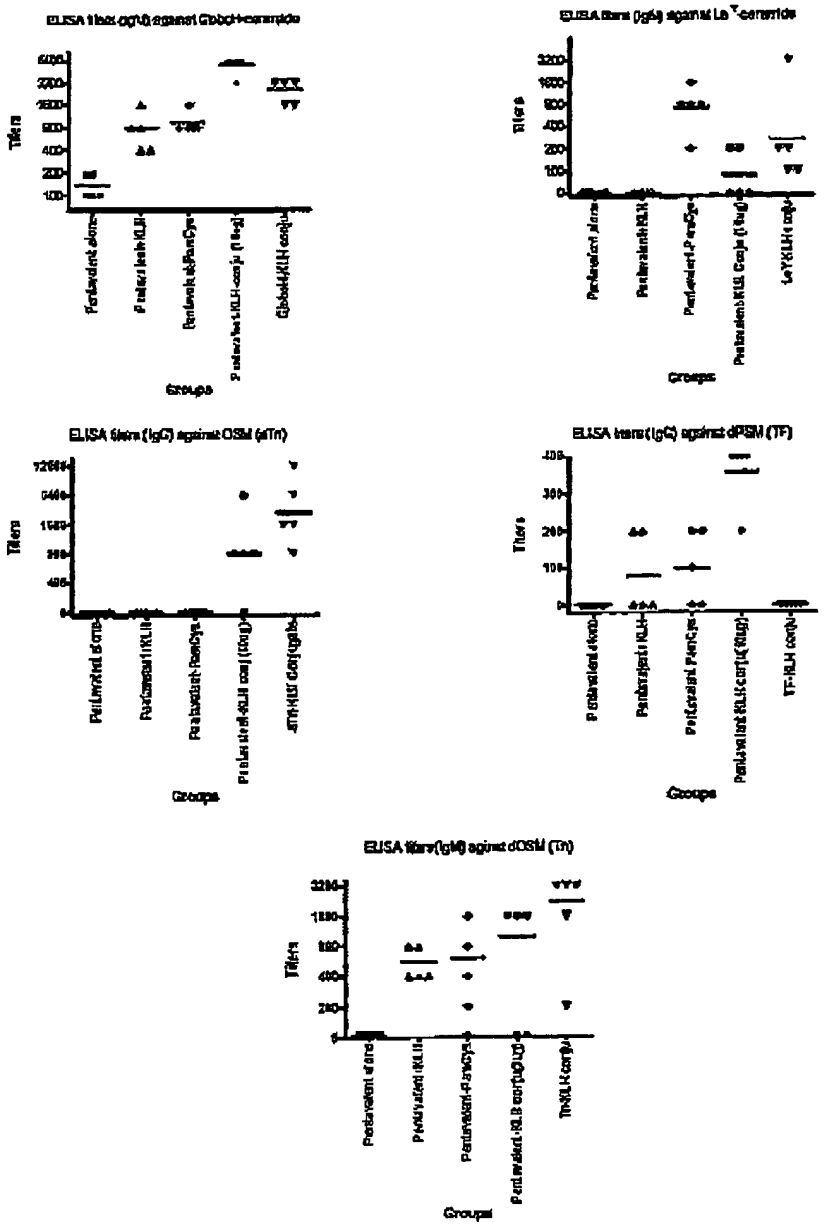


Figure 6. IgM or IgG antibody reciprocal titers after vaccination with different constructs and tested on targets for the five antigens in the pentavalent vaccines. Each data point represents the peak titer for an individual mouse after immunization, and the horizontal lines indicate the mean for the group of five mice. ELISA assays were performed to determine IgM and IgG serum antibody titers as previously described.[7] In brief, Globo-H ceramide, Lewis^y ceramide, ovine submaxillary mucin (OSM expressing sTn), desialylated ovine submaxillary mucin (dOSM expressing Tn), or desialylated porcine submaxillary mucin (dPSM expressing TF) was coated on ELISA plates at an antigen dose of 0.1−0.2 μg/well and incubated overnight at 4 °C. Nonspecific sites were blocked with 3% human serum albumin (HSA) for 2 h, and serially diluted antiserum was added to each well. After 1 h of incubation, the plates were washed, and alkaline phosphatase labeled goat anti-mouse IgM or IgG was added at 1:200 dilution (Southern Biotechnology Associates Inc., Birmingham, AL). The antibody titer was defined as the highest dilution with absorbance of 0.1 or greater over that of normal control mouse sera.

gens were synthesized by the logic of glycal assembly. Among the methodologies previously developed in our laboratory in support of glycal assembly were the use of glycal epoxides and protocols which achieve sulfonamidoglycosylation in either two or three steps from glycals. The approaches for spanning the molecular space between the individual antigens included the use of norleucine spacers, spacers built through Wittig-like reactions followed by asymmetric hydrogenation, and spacers engineered via cross-metathesis. The homogeneous multiantigenic systems were assembled by peptide bond constructions and coupled covalently to KLH carrier protein. Extensive preclinical investigations in mice, including ELISA assays and

Table 1. Reciprocal Median Peak ELISA Titer after Immunization of Groups of Five Mice with the Indicated Antigenic Constructs Plus 10 μg of QS-21

		Globo H-ceramide		Le^y-ceramide		STn (OSM)		TF (dPSM)		Tn (dOSM)	
vaccine	amount injected	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
pooled monovalent-KLH conjugates[a]	3 μg each	800	0	400	0	0	0	0	0	3200*	0
pentavalent-KLH conjugate (2)	10 μg	6400*	400*	0	0	0	800*	200*	400*	200	0
pentavalent (1)	10 μg	100	0	0	0	0	0	0	0	0	0
pentavalent (1) + KLH	10 μg	400	0	100	0	0	0	0	0	400	0
pentavalent-PamCys conjugate (3)	30 μg	800	0	800	0	0	0	0	100	800	0
KLH	10 μg	400	0	0	0	0	0	0	0	100	0
QS-21 alone	10 μg	200	0	0	0	0	0	0	0	0	0

[a] Globo-H-KLH, Le^y-KLH, STn-KLH, TF-KLH, and Tn-KLH. The data presented represent the median of the antibody responses obtained from five mice. On the basis of an examination of the titers obtained from the individual mice within each group, we identified the instances where the titer values are significantly different from all other groups ($p < 0.05$ by the two sample ranks test). In each case, an asterisk is placed next to the significantly superior titer value.

Table 2. Median Percentage Positive Cancer Cells (Mean Fluorescent Intensity) after Immunization of Groups of Five Mice with the Indicated Antigenic Constructs Plus QS-21

		tumor cell line; antigens expressed by the cell line					
		MCF-7; Globo-H, Le^y, STn, TF, Tn		LSC; Le^y, STn, Tn		DU-145; Le^y, TF	
vaccine	amount injected	IgM	IgG	IgM	IgG	IgM	IgG
pooled monovalent-KLH conjugates[a]	3 μg each	23(162)	45(199)	35(70)	34(30)	38(300)	62(101)
pentavalent-KLH conjugate (2)	10 μg	6(102)	23(222)	9(158)	53(175)	35(114)	33(97)
pentavalent (1)	10 μg	4(82)	4(73)	2(107)	2(34)		
pentavalent (1) + KLH	10 μg	10(109)	40(235)	14(51)	59(42)		
pentavalent-PamCys conjugate (3)	30 μg	30(183)	4(59)	41(101)	3(14)	8(70)	4(47)
KLH	10 μg	9(126)	10(175)	9(172)	10(114)		
QS-21 alone	10 μg	4(89)	3(62)	3(116)	2(37)		

[a] Globo-H-KLH, Le^y-KLH, STn-KLH, TF-KLH, and Tn-KLH.

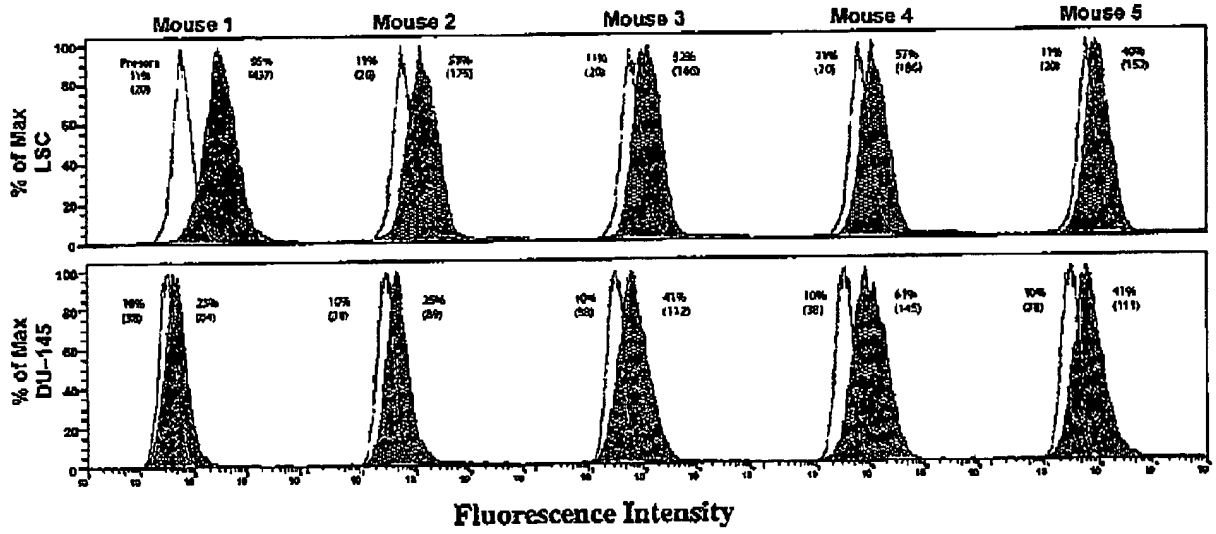


Figure 7. IgG FACS profiles for the five mice immunized with 10 μg of unimolecular pentavalent-KLH vaccine (2) plus QS-21 tested on LSC colon cancer and DU-145 prostate cancer cell lines. Median pre- and postvaccination results shown, % positive cells (MFI). FACS analysis (v(a)): MCF-7 human breast cancer cells expressing all five antigens (but especially globo H), LSC expressing especially Lewis Y, Tn, and sTn, and DU-145 expressing especially Lewis Y and TF were used. Single-cell suspensions of 5×10^7 cells/tube were washed in phosphate-buffered saline with 3% fetal calf serum and incubated with 20 μL of 1/200 diluted antisera for 30 min on ice. A total of 20 μL of 1/15 goat anti-mouse IgG or IgM labeled with FITC was added, and percent positive cells and mean fluorescent intensity (MFI) of stained cells were analyzed using a FACScan (Becton Dickinson, San Jose, CA). Pre- and postvaccination sera were analyzed together, and the pretreatment percent positive cells gained at 10%. Results were considered positive when percent positive cells was 2-fold the negative controls (>20% positive cells) and the MFI was 150% or more of the negative control MFI.

FACS analysis, were conducted on the constructs 1−3. The cumulative data suggest that the immunological properties of the individual antigens are preserved in these highly elaborate vaccines and constitute a strong case for evaluating the central concept of the unimolecular multiantigenic fully synthetic vaccine in the setting of clinical trials. The conjugation and

immunological testing of the newly prepared multiantigenic vaccines **12** and **13** will be reported in due course.

Acknowledgment. This work was supported by the National Institutes of Health Grant CA28824 (to S.J.D.) and by Grants from the Breast Cancer Research Foundation and the NIH (CA052477) to P.O.L. Y.S.C. is grateful for an Army Prostate Cancer Fellowship.

Supporting Information Available: Experimental procedures and characterization data for compounds **12**, **13**, and **16**–**22**. This material is available free of charge via the Internet at http://pubs.acs.org.

JA057244+